

Candidacidal Factors in Murine Bronchoalveolar Lavage Fluid

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Received 4 August 1986/Accepted 11 November 1986

Respiratory secretions provide an efficient method for protecting the large surface area of the lower respiratory tract. To determine whether lung secretions contribute to antifungal defenses, we tested bronchoalveolar lavage fluid for fungicidal activity. *Candida albicans* (blastospores) was incubated in unconcentrated cell-free lavage fluid from Swiss Webster mice and then cultured quantitatively to measure residual viability. In control buffer the residual fractions of viable fungi were 1.03 ± 0.12 at 60 min and 0.84 ± 0.05 at 120 min, whereas the residual fractions in lavage fluid were 0.64 ± 0.07 and 0.23 ± 0.05 , respectively ($P < 0.05$ by *t* tests). This activity was trypsin sensitive and heat stable (56°C) and did not require divalent cations. It did not sediment with the surfactant fraction of lung lavage fluid. Unconcentrated lavage fluid reduced the adherence of *C. albicans* to serum-coated glass tubes to $2.3 \pm 1.5\%$ of that of control *Candida* suspensions ($n = 5$, $P < 0.05$ by *t* test). It did not alter *Candida* ingestion or intracellular processing by alveolar macrophages. Lavage fluid also killed clinical isolates of *Candida tropicalis* and *Torulopsis glabrata* but did not kill *Candida krusei* or *Candida parapsilosis*. Lavage fluid was concentrated and passed through an acrylamide-agarose gel matrix. The chromatogram indicated that the candidacidal activity eluted in a peak with a molecular weight range of 29,000 to 40,000. After electrophoresis on 15% sodium dodecyl sulfate-polyacrylamide gels, these fractions resolved into three bands. These were transferred to nitrocellulose and then eluted with Triton X-100; this procedure permitted the isolation of a single band of candidacidal activity with a molecular weight of 29,000. In summary, murine lavage fluid contains a heat-stable protein with direct antifungal activity. This soluble factor may contribute to lung defense processes by reducing fungal viability and adherence to tissue surfaces.

Candida albicans infections frequently develop in patients with a variety of acute and chronic diseases which impair host defense responses, and these infections produce significant morbidity and mortality (6). Pulmonary infections with *C. albicans* may develop in these patients after aspiration of oropharyngeal flora or through hematogenous dissemination from remote infections (13). Since oropharyngeal candidiasis is extremely common in immunosuppressed patients and should represent an important risk factor in the development of *Candida* pneumonia (2, 5), we have used a murine model to investigate pulmonary defense responses against *C. albicans* (16). These studies demonstrated that large *Candida* inocula (5×10^5 blastospores per mouse) elicited a neutrophil influx into lung tissue and bronchoalveolar spaces and that after this influx viable *C. albicans* cells were eliminated from the lower respiratory tract. In subsequent studies we found that smaller *Candida* inocula (1,000 blastospores per mouse) were also cleared from the lung but did not elicit a neutrophil influx (unpublished data). These latter results implied that intrinsic pulmonary defenses could protect the lung from modest *Candida* challenges. Since pulmonary macrophages have relatively weak candidacidal activity in vitro (11; unpublished data), we considered the possibility that soluble factors in the lower respiratory secretions have direct candidacidal activity. The results in this paper describe the isolation and partial characterization of a candidacidal protein in murine lavage fluid.

MATERIALS AND METHODS

Mice. Outbred female Swiss Webster mice (20 to 25 g) were used throughout these experiments. In a few experiments athymic BALB/c mice (both homozygotes and heterozygotes) were used.

***Candida* strains.** Clinical isolates of *C. albicans* and other *Candida* species were obtained from The University of Iowa Hospitals and Clinics Clinical Microbiology Laboratory, Iowa City, and were identified by germ tube formation and sugar assimilation tests. These isolates were maintained on brain heart infusion agar plates and were grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) as needed.

Bronchoalveolar lavage. Mice were sacrificed with air emboli, and the trachea was exposed and cannulated with a blunt 20-gauge needle. Buffered solution (1 ml) was injected and withdrawn three times and then removed completely. Recoveries ranged from 60 to 80%. As described in the Results section several buffers, including phosphate-buffered saline (PBS) (pH 7.4), PBS supplemented with 1.25 mM EDTA (pH 7.4), and 0.9% saline, were used in these experiments. Macrophages and large particles were removed by centrifugation ($150 \times g$ for 10 min), and cell-free lavage fluid was used for the assays described below.

Candidacidal assay. Overnight cultures of *C. albicans* ($>98\%$ blastospores) were washed with PBS (pH 7.4) and resuspended in PBS. Samples of these suspensions were then diluted into lavage fluid or buffer to produce a final yeast concentration of 1×10^6 to 2×10^6 /ml. These mixtures were then incubated at 37°C and cultured quantitatively at 0, 60, and 120 min to determine the number of viable yeasts.

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For these quantitative cultures 100- μ l samples were spread with glass rods on the agar surface. In each experiment the number of viable yeasts at 60 and 120 min was expressed as a fraction of the number at zero time. After the initial experiments investigating the time course for loss of viability, all candidacidal activities were determined with a 120-min incubation period.

***Candida* adherence assays.** Glass Leighton tubes were incubated with tissue culture medium (Eagle minimum essential medium with 10% heat-inactivated newborn calf serum [GIBCO Laboratories, Grand Island, N.Y.]) for 60 min. Then suspensions of *C. albicans* incubated in either PBS or PBS lavage for 30 to 120 min (conditions identical to the candidacidal assay described above) were added to the Leighton tubes and incubated for 60 min at 37°C. These Leighton tubes were rinsed twice with PBS and fixed with 1% glutaraldehyde in PBS. The number of *C. albicans* adherent to the Leighton tubes was determined with a phase-contrast microscope which had a grid in one eyepiece. Duplicate tubes were included in each assay, and all tubes were coded before microscopic examination. Results were expressed as a percentage of concurrent control blastoconidia incubated in buffer alone.

***Candida* ingestion and intracellular killing.** (i) **Alveolar macrophages.** Mice were sacrificed with air emboli, the tracheas were cannulated with a 21-gauge needle, and each mouse was lavaged with 3 volumes of PBS (0.7 ml). Macrophages were collected by centrifugation ($150 \times g$ for 10 min) and suspended in tissue culture medium consisting of Eagle minimum essential medium, 10% heat-inactivated newborn calf serum, and antibiotics. Cell suspensions were incubated in Leighton tubes containing glass cover slips at 37°C in a 5% CO₂-95% air atmosphere. After 60 to 90 min loose cells and debris were removed with tissue culture medium rinses, and cultures were incubated overnight. Leighton tubes were rinsed again and then incubated for an additional 24 h in fresh tissue culture medium before use in phagocytosis and killing assays.

(ii) ***Candida* ingestion.** Amphotericin B (1 μ g/ml, final concentration) was added to overnight cultures of *C. albicans*, and these cultures were held at 37°C for 60 min. The *Candida* blastoconidia were washed once with PBS, adjusted to the desired concentration, and incubated with mouse lavage fluid (final lavage dilution in incubation mixture, 1:4) for 30 min at 37°C. (Tubes were vortexed every 10 min during this step.) This suspension was then diluted with an equal volume of Eagle minimum essential medium (without serum) and checked for agglutination (which was always absent). These lavage-treated yeasts were then added to washed macrophage monolayers for 30 min. The cover slips were washed twice with medium, air dried, fixed with ethanol, and stained with Giemsa stain. These cover slips were mounted, coded, and examined by light microscopy ($\times 400$) to determine the percentage of the macrophages with intracellular yeasts and the average number of yeasts in these macrophages. These results were used to calculate the number of yeasts per 100 macrophages. Each experiment included duplicate cover slips for control and lavage-treated yeasts. The yeast-to-macrophage ratio for these experiments was approximately 5/1. In pilot experiments we determined that brief exposure to low concentrations of amphotericin B did not kill the *C. albicans* but did significantly reduce adherence to glass during the ingestion step. Consequently, there were only rare extracellular *Candida* yeast forms present, and this simplified the measurement of ingestion by a microscopic technique.

(iii) **Intracellular killing.** After the ingestion steps described above, monolayers were incubated for an additional 180 min and then stained. Using the morphological criteria of Lehrer et al. (11), we determined the number of viable and nonviable intracellular yeasts and the number with germ tubes.

Column chromatography. Whole murine lavage fluid was concentrated to a volume representing 10% of the original volume at 4°C by positive-pressure ultrafiltration (Amicon Corp., Scientific Systems Div., Danvers, Mass.). Filtration membranes with 10,000- and 30,000- M_r cutoffs (PM10 and PM30 Diaflo Ultrafilters, respectively; Amicon) and 40-lb/in² nitrogen were used. A polyacrylamide agarose gel (exclusion limit of 70,000 daltons [Da] [AcA 54, Ultrogel; LKB, Bromma, Sweden]) column (2 cm² by 35 cm) was prepared in 0.02 M Tris hydrochloride buffer, pH 7.6. The void volume and linear fractionation range of the gel column were determined with 5.0% dextran blue 2000 (2×10^6 Da; Pharmacia Fine Chemicals, Piscataway, N.J.) and molecular weight standards. The standard proteins (Sigma Chemical Co., St. Louis, Mo.) selected included: bovine insulin (5,000 Da), cytochrome *c* (12,000 Da), lactalbumin (14,000 Da), soybean trypsin inhibitor (24,000 Da), ovalbumin (45,000 Da), and human serum albumin (68,000 Da). A sample of the concentrated mouse lavage fluid containing candidacidal activity was applied to the top of the gel bed, and the flow rate was adjusted to 6 to 8 ml/h by hydrostatic pressure (14 to 16 cm of H₂O). Eluate fractions of 1.4 ml were collected, and the elution position of candidacidal activity was determined by assaying each successive fraction as described above.

Immunodiffusion and electrophoresis. Candidacidal activity partially purified by ultrafiltration and column chromatography was examined by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, immunoelectrophoresis, and double-diffusion immunoprecipitation analyses. Vertical slab gel electrophoresis was performed with an electrophoresis cell with standard glass plates (18 by 16 cm) and 1.5-mm spacers (Bio-Rad Laboratories, Richmond, Calif.). A 10.0-cm lower separating gel with a 15% polyacrylamide concentration in a 0.1% SDS-0.375 M Tris hydrochloride (pH 8.8) buffer and a 1.0-cm upper stacking gel of 3.0% polyacrylamide were used (8). A running buffer made of 0.025 M Tris hydrochloride, 0.19 M glycine, and 0.1% SDS was used, and electrophoresis was performed at constant current (30 mA) to bring the leading front to within 1 cm of the bottom of the gels. Gels were stained with 0.1% Coomassie brilliant blue containing 0.1% cupric acetate or with 0.1% amido black in 45% (vol/vol) methanol-10% acetic acid or were examined with a photographic amplification system (Kodavue; Eastman Kodak Co., Rochester, N.Y.).

To screen for the presence of a protein in the active fraction with antigenic reactivities similar to those of serum proteins, we performed immunoelectrophoresis using 1.2% agar (low ion; Difco Laboratories, Detroit, Mich.) in 0.03 M barbital buffer (pH 8.1) (7, 14, 22). Double-immunodiffusion precipitation tests were performed in Noble agar (Difco) in borate saline (18). Goat antiserum specific for mouse immunoglobulins, albumin, and mouse whole serum were obtained from Cooper Biomedical, Inc. (West Chester, Pa.).

Gel transfer and protein elution. Candidacidal activity recovered from AcA 54 columns was subjected, in triplicate, to electrophoresis in one-dimensional SDS-polyacrylamide gels as described above. One electrophoresis column was cut from the gel, fixed and stained with 0.1% Coomassie blue containing 0.1% cupric acetate. The proteins in the remaining two columns were then transferred to nitrocellulose

sheets by a modification (8) of the method of Towbin et al. (21). A sheet of nitrocellulose with a 0.45- μ m pore size was cut from a role (Millipore Corp., Bedford, Mass.), wetted with transfer buffer (25 mM Tris hydrochloride, 0.19 mM glycine in 20% [vol/vol] methanol, pH 8.3), and laid in Bio-Rad Transblot apparatus with the SDS-polyacrylamide gel containing the proteins to be transferred. The gel-nitrocellulose assembly was then placed into a transfer assembly with the gel placed anodally in relation to the nitrocellulose. Electrophoresis proceeded at 45 V for 3 h. Again, the blotted polyacrylamide gels were fixed and stained with Coomassie blue. The nitrocellulose sheet containing the partially purified murine lavage proteins, which had been electrophoretically transferred in duplicate, was cut vertically, separating the electrophoretic columns. One strip of nitrocellulose was stained with amido black, and the second strip was preserved in transfer buffer at 4°C.

Proteins from single electrophoretic bands were eluted from nitrocellulose with a nonionic detergent (0.002 M Triton X-100 in Tris hydrochloride, pH 7.8). Nitrocellulose strips containing proteins which had been electrophoresed and then transferred in duplicate were placed side by side: one was stained with 0.1% amido black, revealing several distinct bands; a second was preserved in transfer buffer at 4°C for less than 120 min. The unstained strip of nitrocellulose paper was then cut to isolate protein bands revealed on the stained strip. The relative positions of the newly separated paper pieces were noted. Each was placed into a polystyrene tube with 2.5 ml of nonionic detergent, and the tubes were tumbled at 4°C for 16 h. Samples of each of the elution solutions were then analyzed for candidacidal activity as described above. The concentration of total protein in the eluted solution was determined by the method of Lowry et al. (12).

Chemical reagents. Concanavalin A and mannan (*Saccharomyces cerevisiae*) were obtained from Sigma Chemical Co.

Surfactant preparation. Mouse lungs were lavaged with saline, and this lavage fluid was clarified by low-speed centrifugation ($150 \times g$ for 10 min). The surfactant fraction was then collected by high-speed centrifugation ($40,000 \times g$ for 30 min), and the pellet was suspended in 16% NaBr. This solution was then overlaid with 13% NaBr which was in turn overlaid with 0.9% NaCl (20). This discontinuous gradient was spun at $116,000 \times g$ for 120 min. The surfactant factor was collected from the top layer by centrifugation ($40,000 \times g$ for 30 min) and suspended in PBS (at one-half the original lavage volume).

Chloroform-methanol extraction. Unconcentrated lavage fluid was mixed with a chloroform-methanol solution (2:1, vol/vol) and agitated. The layers were separated by low-speed centrifugation ($150 \times g$ for 10 min). The chloroform layer was dried down under N_2 , and the residue was suspended in PBS. The methanol layer was dialyzed against PBS and concentrated with an Amicon filter.

Data analysis. All results in the text, figures, and tables are presented as the mean \pm 1 standard error. Results were analyzed for differences by *t* tests, paired *t* tests, and analysis of variance. A *P* value of ≤ 0.05 was considered significant.

RESULTS

Candidacidal activity. In the initial experiments we used PBS supplemented with 1.25 mM EDTA (pH 7.4) for bronchoalveolar lavage. Unconcentrated cell-free lavage fluids killed *C. albicans* during incubation at 37°C (Fig. 1).

Candida agglutination did not occur during this incubation period (phase-contrast microscopy, $\times 400$ magnification), and *C. albicans* did not adhere to the Pyrex tubes used in these assays (examined by phase-contrast microscopy). We also used assay mixtures with lower concentrations of *C. albicans* to minimize any possible agglutination during the incubation step. These results demonstrated that lavage fluid was also active under these conditions and that the residual fractions at 120 min with 10^5 , 10^4 , and 10^3 blastoconidia per ml of assay mixture were 0.43, 0.28, and 0.31, respectively ($n = 4$).

We determined that lower respiratory tract secretions recovered with PBS (pH 7.4) and with 0.9% saline killed *C. albicans* in similar assays. This candidacidal activity was active at pH 6.0 to 8.0 in PBS. The addition of either 1 mM Ca^{2+} or 1 mM Mg^{2+} did not significantly alter this fungicidal effect. Lavage fluids diluted four- to eightfold contained sufficient candidacidal activity to kill 50% of the standard inocula.

Other laboratory animals. Unconcentrated lavage fluid recovered from athymic BALB/c mice, both homozygotes and heterozygotes, also killed this *C. albicans* isolate. The residual fractions of viable *C. albicans* after 120 min of incubation were 0.10 (homozygotes, $n = 2$ determinations) and 0.24 (heterozygotes, $n = 3$ determinations). In addition, lavage fluid from NBR rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) and rabbits (outbred white) also killed this isolate (residual fraction = 0.21, $n = 3$ determinations for rats; residual fraction = 0.15, $n = 1$ for rabbits).

Other *Candida* species. Unconcentrated lavage fluid from Swiss Webster mice killed an isolate of *C. tropicalis*, *Torulopsis glabrata*, and a second *C. albicans* isolate but did not kill *C. parapsilosis*, *C. krusei*, and a third isolate of *C. albicans* (Table 1).

Characteristics of candidacidal factor. As stated above, the candidacidal factor was active in 1.25 mM EDTA and was

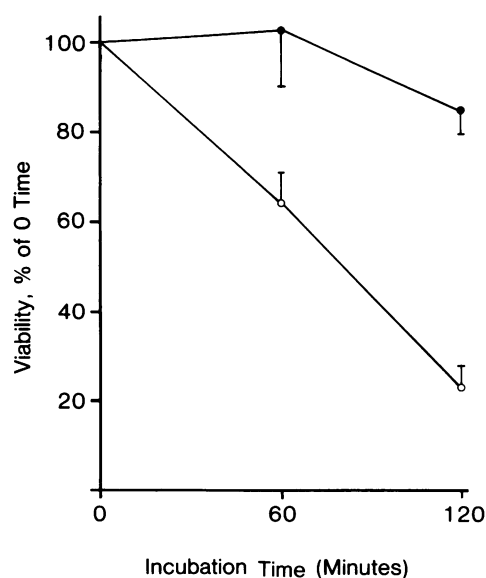


FIG. 1. *Candida* viability in unconcentrated lavage fluid. *C. albicans* blastoconidia were incubated in PBS-EDTA lavage fluid or PBS-EDTA buffer for 120 min. The residual fraction of viable *C. albicans* is plotted against time for control buffer (●) and lavage fluid (○). The differences at 60 min ($n = 6$) and 120 min ($n = 8$) are both significant by *t* tests ($P < 0.05$).

TABLE 1. Candidacidal activity against a panel of *Candida* species

Clinical isolate	Residual fraction ^a
<i>Candida albicans</i> no. 2	2.12 ± 0.52
<i>Candida albicans</i> no. 3	0.41 ± 0.22
<i>Candida krusei</i>	1.22 ± 0.26
<i>Candida tropicalis</i>	0.11 ± 0.11
<i>Candida parapsilosis</i>	1.07 ± 0.14
<i>Torulopsis glabrata</i>	0.25 ± 0.13

^a Residual fractions (mean ± standard error) for three to five experiments per isolate, in which the residual fraction is the ratio of CFU after 120 min of incubation to the CFU after 0 min of incubation.

not influenced by the addition of either Ca^{2+} or Mg^{2+} PBS. This factor was heat stable (56°C for 30 min), trypsin sensitive (250 $\mu\text{g}/\text{ml}$ for 30 min at 37°C), and did not sediment with the surfactant fraction during high-speed centrifugation (36,000 × g for 20 min) (Table 2). After a chloroform-methanol extraction procedure the active material was recovered in the methanol phase. Candidacidal activity was not recovered in surfactant purified on sodium bromide gradients (residual fractions = 1.04 for buffer control and 0.99 for surfactant preparations, $n = 2$).

***Candida* permeability.** We used rifampin susceptibility as an index of *Candida* permeability (15). Suspensions of *Candida* blastoconidia were incubated in diluted lavage fluid (one-fourth the usual concentrations) for 0 and 120 min and then plated on Trypticase soy broth plates with and without rifampin (5 $\mu\text{g}/\text{ml}$). There was no additional rifampin effect on *Candida* killing (the residual fractions on control plates = 0.63 and those on the rifampin plates = 0.63, $n = 3$). We used a lower concentration of lavage fluid in these experiments to facilitate detection of any synergistic interaction.

Inhibitors. Concanavalin A (10 $\mu\text{g}/\text{ml}$, final concentration) did not inhibit candidacidal activity in lavage fluid ($n = 3$). Mannan (1 mg/ml) did partially inhibit this activity (residual fractions: control, 0.36 ± 0.12; mannan, 0.48 ± 0.13; $n = 4$, $P < 0.05$ by paired t test). Serum (1% heat-inactivated newborn calf serum) did not alter the in vitro killing ($n = 3$).

Lavage effects on *Candida* adherence capacity. *C. albicans* suspensions (10⁶/ml) were incubated with PBS or lavage fluid for 30 to 120 min at 37°C and then incubated in serum-coated glass Leighton tubes. Lavage fluid exposure for 60 and 120 min significantly reduced *Candida* adherence (Fig. 2).

Lavage effects on macrophage functions. Pretreatment of

C. albicans yeast forms with lavage fluid (one-fourth of the concentrations used for viability determinations) did not alter *Candida* ingestion by alveolar macrophages. In control monolayers there were 20.7 ± 8.2 intracellular yeasts per 100 macrophages, whereas in monolayers exposed to pretreated *C. albicans* there were 20.4 ± 6.8 intracellular yeasts per 100 macrophages ($n = 6$). In addition, pretreatment with lavage fluid did not alter intracellular processing. In control monolayers, 8.5 ± 2.0% of intracellular yeasts had germ tubes, and 68.5 ± 1.9% were dead by morphological criteria. In monolayers exposed to *C. albicans* pretreated with lavage fluid 6.5 ± 3.0% of the intracellular yeasts had germ tubes, and 71.0 ± 3.5% were dead ($n = 4$ experiments).

Molecular weight and charge characteristics. Candidacidal activity was fully retained by a 10,000- M_r cutoff ultrafiltration membrane. The same activity was only partially retained by a molecular sieve with a 30,000- M_r cutoff (75% retained). Identical 30,000- M_r cutoff membranes retained 95% of human serum albumin (67 kDa) used as a molecular weight marker and retained 75% of α -chymotrypsinogen (24.5 kDa).

Column chromatography with the acrylamide-agarose matrix yielded linear fractionation for proteins with molecular weights of 12,000 to 68,000. The elution volume in which standard proteins appeared graphed as a function of the common logarithm of molecular weight closely approximated a straight line ($r = 0.94$). Candidacidal activity eluted reproducibly in volumes of 26 to 29 ml which contained proteins with molecular weights of 25,000 to 40,000. Electrophoresis of the chromatography fraction containing the candidacidal activity in 15% SDS-polyacrylamide gels resulted in three protein bands (Fig. 3). Two electrophoretic bands were particularly prominent: a sharp, well-delineated band with a molecular weight of 45,000 and a broader band containing proteins with a molecular weight of 29,000. A

TABLE 2. Characteristics of candidacidal activity

Expt	Residual fraction after 120 min of incubation (mean ± SE)
Control buffer	0.86 ± 0.05 ($n = 5$)
Buffer + trypsin ^a	0.97 ± 0.09
Lavage	0.29 ± 0.12
Lavage + trypsin ^a	0.83 ± 0.04
Control buffer	0.91 ± 0.05 ($n = 6$)
Lavage	0.31 ± 0.11
Lavage (56°C)	0.32 ± 0.12
Control buffer	0.97 ± 0.28 ($n = 5$)
Lavage	0.12 ± 0.11
Pellet ^b	0.53 ± 0.16
Supernatant	0.06 ± 0.05

^a Trypsin (250 $\mu\text{g}/\text{ml}$) for 30 min at 37°C; then *C. albicans* was added.

^b Pellet from high-speed centrifugation (36,000 × g for 20 min).

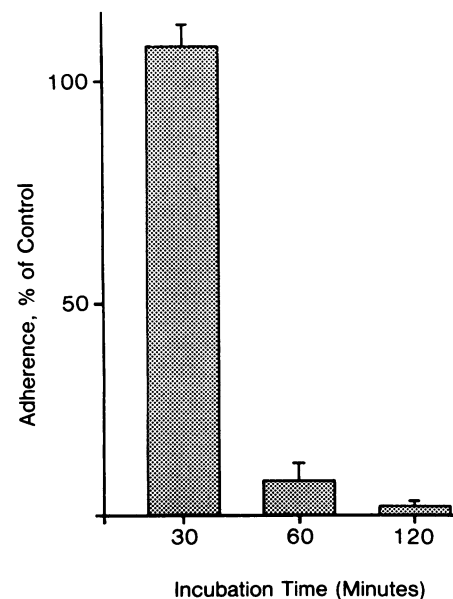


FIG. 2. Lavage effects on *Candida* adherence to serum-coated glass. *Candida* suspensions were incubated in buffer or lavage fluid for 30 to 120 min and then incubated in serum-coated glass Leighton tubes. Results represent three to five separate experiments. There is a significant reduction in adherence capacity after 60 and 120 min of incubation in lavage fluid (by paired t tests).

much less distinct protein in this chromatography fraction electrophoresed with a molecular weight of 35,000.

Attempts at electrophoretic transfer from polyacrylamide gels to nitrocellulose with the gel cathodal to the paper were unsuccessful. Reversing the position of the gel-nitrocellulose sandwich in the transfer chamber so that the proteins in the gel were anodal to the nitrocellulose paper resulted in successful transfer of the candidacidal activity. Amido black stains of the transferred proteins suggested that the proteins were not quantitatively transferred to the nitrocellulose, because the previously prominent 45,000-Da protein was much less prominent after transfer. The 29,000-Da protein remained broad and stained very boldly. Separation of the 29,000-kDa blot from the narrow 35,000-Da protein and elution in a nonionic detergent resulted in a solution with a protein concentration of 0.1 mg/ml. The candidacidal activity was localized to this 29,000-Da band. Triton (0.002 M) used as a control in these experiments did not have candidacidal activity, and the addition of Triton X-100 to blastoconidia treated with unfractionated lavage did not facilitate killing ($n = 3$).

Serum identity. Double immunodiffusion and immunoelectrophoresis were used to analyze the column chromatography fraction and the transblotted protein with candidacidal activity. When goat antisera with specificity for the mouse immunoglobulin F(ab')₂ and Fc γ , α , and μ regions were applied, no immunoprecipitin arcs were evident.

DISCUSSION

These experiments demonstrate that murine lavage fluid contains a protein with direct candidacidal activity. Unconcentrated lavage fluid also reduced *Candida* adherence to serum-coated plastic surfaces, but it did not have either opsonic or procidal activity when tested in vitro with alveolar macrophages. This soluble factor in lung secretions probably contributes to antifungal defenses when lower numbers of *C. albicans* reach the lower respiratory tract.

Other investigators have described extracellular factors which potentially contribute to host defenses against *Candida* species. LaForce et al. (10) isolated two *Candida*-agglutinating activities from rabbit lavage fluid. One of these activities was a 10,000-Da protein which was recovered in the phospholipid-rich fraction of lavage fluid; the other was immunoglobulin A. These workers suggested that yeast agglutination facilitates the extracellular fungicidal activity of alveolar macrophages, but the exact role of agglutination in host defenses remains uncertain. In these experiments we carefully checked for agglutination and did not find any. We subsequently used higher concentrations of *Candida* blastoconidia (2×10^7 /ml) to detect agglutination and did observe agglutination. However, unlike the candidacidal activity, agglutination was completely inhibited by EDTA (K. M. Nugent, K. Couchot, and D. Nash, Am. Rev. Respir. Dis. 133:A237, 1986). Pollock and co-workers (18) have isolated histidine-rich polypeptides from human parotid secretions which have fungistatic and fungicidal activity against *C. albicans*. These polypeptides had growth inhibition and killing effects at concentrations of 25 μ g/ml and had significant effects on *Candida* permeability measured by potassium release. These salivary proteins probably protect oral surfaces from *Candida* colonization. These workers also demonstrated that high concentrations of hen egg white lysozyme inhibited *C. albicans* growth. We tested commercial lysozyme against this *Candida* isolate and did not observe any killing activity. Human lactoferrin (in an iron-

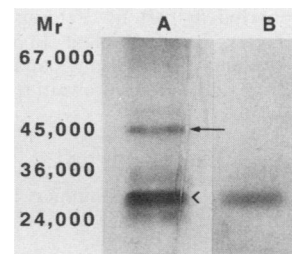


FIG. 3. Polyacrylamide gel electrophoresis and electrophoretic transfer of candidacidal activity. Partially purified activity derived chromatographically was run in a 15% acrylamide slab gel and stained with Coomassie blue (lane A). A sharp, narrow 45,000-Da band (arrow) and a broader 29,000-Da protein (arrowhead) are present; less distinct is a 35,000-Da protein. Lane B displays the electrophoretic blot of the 29,000-Da cationic protein onto a nitrocellulose paper strip (amido black stain).

free state) also has potent candidacidal activity (1). This protein is synthesized by neutrophils and acinar epithelial cells and has been detected in secretions from several mucosal surfaces. The activity we described has a lower molecular weight than lactoferrin and transferrin (76,000 to 81,000).

Lehrer and co-workers (17, 19) have isolated cationic proteins from rabbit alveolar macrophages and rabbit peritoneal exudate leukocytes which have direct candidacidal activity. The most active leukocyte peptides (at concentrations of 2.5 μ g/ml) killed 99% of a *Candida* suspension within 20 min. These peptides function best in low-ionic-strength buffers and against *C. albicans* in log-phase growth. Ca^{2+} but not Mg^{2+} stimulated killing. Peptides from macrophages inhibited O_2 consumption and increased cell permeability (17). Whether or not these macrophage peptides are released and function in vivo is unknown. The activity we identified has a higher molecular weight, is active in isotonic buffer, and therefore is unlikely to be related to these lysosomal cationic proteins.

Coonrod and his associates (3, 4) have recently demonstrated that cell-free rat lavage has potent bactericidal activity, especially against *Streptococcus pneumoniae*. They recovered this activity in the surfactant fraction and demonstrated that the free fatty acids were responsible for the killing. These substances activated the pneumococcal autolysin (which caused cell lysis) and also caused direct increases in permeability (4). Laforce and Boose (9) reported that lung lavage contains a peptide which causes sublethal damage to *Escherichia coli*. This peptide had a low molecular weight and required a metal cofactor (9). These studies with bacterial pathogens indicate that the lung has potent noncellular defenses against microbes. We suggest that these various activities in respiratory secretions provide basal protection against low-intensity challenges and help maintain the relative sterility of the lower respiratory tract.

In summary, murine bronchoalveolar lavage fluid contains a heat-stable cationic protein with direct candidacidal activity. This protein does not require divalent cations and works well in isotonic solutions. These results and other reports (3, 4, 9) suggest that extracellular factors have an important role in lung defense processes.

ACKNOWLEDGMENTS

We thank Robert Anderson, Sue Squier, and Steven Strickler for assistance with these experiments.

This work was supported in part by Veterans Administration research funds (K.M.N.), by a Clinical Investigator award (HL01065) from the National Heart, Lung, and Blood Institute (R.B.F.), and by an E. L. Trudeau Fellow award from the American Lung Association (R.B.F.).

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